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That I am knowledgeable in the English language and in the language in which the below identified application was filed, and that I believe the English translation of the Japanese Patent Application No. 224187/1998 is a true and complete translation of the above-identified Japanese Patent Application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

September 21, 2004

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Applicant(s): CHUGAI SEIYAKU KABUSHIKI KAISHA

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[Title of the Invention] CONJUGATE OF MATRIX METALLOPROTEASE INHIBITOR AND HYALURONIC ACID

[Number of Claims] 12

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[Item] Specification 1

[Item] Abstract 1

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[Name of Document] SPECIFICATION

[Title of Invention]

CONJUGATE OF MATRIX METALLOPROTEASE INHIBITOR AND HYALURONIC ACID

[CLAIMS]

[Claim 1] A covalent conjugate of (1) at least one matrix metalloprotease inhibitor and (2) hyaluronic acid, a hyaluronic acid derivative or a salt thereof.

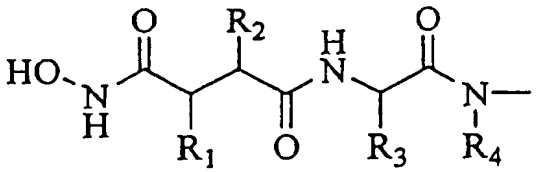
[Claim 2] The covalent conjugate of claim 1, wherein the matrix metalloprotease inhibitor binds to hyaluronic acid, a hyaluronic acid derivative or the salt thereof via a spacer.

[Claim 3] The covalent conjugate of claim 1 or 2, wherein the weight ratio of the matrix metalloprotease inhibitor to the entire covalent conjugate is 0.01 to 50%.

[Claim 4] The covalent conjugate of any one of claims 1 to 3, wherein the matrix metalloprotease inhibitor is a hydroxamic acid residue.

[Claim 5] The covalent conjugate of any one of claims 1 to 4, wherein the matrix metalloprotease inhibitor is a hydroxamic acid residue represented by the general formula (1):

[Formula 1]



(1)

wherein

R_1 is a hydrogen atom, a hydroxyl group or a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_2 is a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_3 is a straight chain or branched alkyl group having 1 to 8 carbon atoms which may be substituted with a cycloalkyl group, an aryl group or a heterocyclic group; and

R_4 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[Claim 6] The covalent conjugate of any one of claims 1 to 5, wherein the spacer is represented by the general formula (2):

$$-R_5-R_6-R_7-R_8- \qquad (2)$$

wherein

R_5 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms;

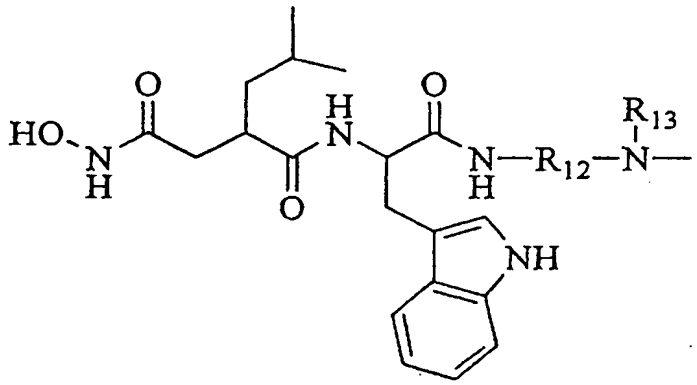
R_6 is an oxygen atom or a methylene or imino group which may be substituted with a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms;

R_7 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms; and

R_8 is an oxygen atom, a sulfur atom or NR_9 wherein R_9 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[Claim 7] The covalent conjugate of any one of claims 1 to 6, wherein the conjugate of the matrix metalloprotease inhibitor and the spacer is represented by the general formula (3):

[Formula 2]



(3)

wherein

R_{12} is a straight-chain or branched-chain alkylene group having 3 to 21 carbon atoms or -(alkylene group having 1 to 8 carbon atoms)-O-(alkylene group having 1 to 8 carbon atoms)-; and

R_{13} is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[Claim 8] The covalent conjugate of any one of claims 1 to 7, wherein the matrix metalloprotease inhibitor in the form of a conjugate with hyaluronic acid, a hyaluronic acid derivative or a salt thereof inhibits a matrix metalloprotease *in situ*.

[Claim 9] A method for preparing the covalent conjugate of any one of claims 1 to 8 comprising binding a

site of the matrix metalloprotease inhibitor that does not affect the activity of the inhibitor to a carboxyl group, a hydroxyl group or a functional group at the reducing end of hyaluronic acid, a hyaluronic acid derivative or a salt thereof by direct chemical reaction or via a spacer.

[Claim 10] A pharmaceutical composition comprising the covalent conjugate of any one of claims 1 to 8.

[Claim 11] The pharmaceutical composition of claim 10 which is a therapeutic agent for joint disease.

[Claim 12] The pharmaceutical composition of claim 11, wherein the joint disease is osteoarthritis, rheumatoid arthritis or scapulohumeral periarthritis.

[Detailed Description of the Invention]

[0001]

[Technical Field to which the Invention Pertains]

The present invention relates to hyaluronic acid, a derivative thereof or a salt thereof, which has matrix metalloprotease inhibitor bound. More specifically, the present invention relates to a covalent conjugate obtained by chemically covalent binding hyaluronic acid, a derivative or a salt thereof to matrix metalloprotease inhibitor which is effective for treating osteoarthritis, rheumatoid arthritis and the like, a method for preparing the conjugate, and a pharmaceutical composition containing the conjugate.

[0002]

[Prior Art]

Articular cartilage is composed of about 70% of water,

chondrocytes and a cartilage matrix. The major components constituting the cartilage matrix are collagen and proteoglycan; the proteoglycan having good water retention characteristics is contained in the network of collagen having a reticulated structure. The cartilage matrix is rich in viscoelasticity and has an important role in reducing the stimulus and load imposed on the cartilage in order to maintain the normal morphology and function of the articular cartilage.

[0003]

Osteoarthritis (hereinafter also referred to as "OA") and rheumatoid arthritis (hereinafter also referred to as "RA") are both representative of the diseases accompanied by the destruction of the cartilage matrix. It is thought that the destruction of the matrix in these diseases is triggered by mechanical stresses with aging in the case of OA and by excess proliferation of the surface layer cells of the synovial membrane, pannus formation and inflammatory cell infiltration in the case of RA, and both phenomena are caused through the induction of proteases. Since the degradation of cartilage matrix is progressed in the extracellular region at a neutral pH, it is said that a matrix metalloprotease (hereinafter also referred to as "MMP" or "MMPs" when used as the general term) whose optimal pH is in the neutral range plays a leading role in the degradation.

[0004]

Up to now, as to humans, 16 types of proteases

which belong to the MMP family have been reported; 4 types of endogenous proteins which bind to the proteases and inhibit their activities have been found and named tissue metalloprotease inhibitors (hereinafter also referred to as "TIMP" or TIMPs" when used as the general term). MMPs exhibit various functions such as genesis, angiogenesis, estrous cycle, bone remodeling and tissue repair in physiological conditions. In order to appropriately exhibit these functions, each step of the production, the activation and the interaction with the substrate of MMPs is strictly controlled by TIMPs etc. In other words, it is thought that the destruction of the matrix in diseased conditions is caused by some failures in the controlling mechanism, resulting in excessive production and activation of MMPs.

[0005]

Therefore, drugs inhibiting MMPs are extremely promising as the drugs suppressing the destruction of cartilage matrix in joint diseases such as OA and RA. Until now, many drugs inhibiting MMPs have been reported; among them, MMP inhibitors which are hydroxamic acids are presently most noted because of their strong inhibiting activity and high specificity to MMPs. Hydroxamic acids exhibiting MMP inhibition even by oral administration have been found and some of which have been already entered into clinical trials on cancer patients and arthritis patients.

[0006]

However, MMP inhibitors of this type have a serious

defect that they more or less show inhibiting activity against all types of MMPs and suppress even the MMPs taking part in physiological functions. In fact, in the clinical trials in progress of hydroxamic acids on patients of cancer, side effects such as skeletal muscle pains have been reported, although they are transient. Recently, improved products having heightened specificity to certain MMPs are under development, however no MMPs involved in diseased conditions alone have been found yet. Furthermore, since novel MMPs are found one after another, there still remains a possibility that some physiological actions of MMPs are suppressed when an MMP inhibitor is systemically administered.

[0007]

The local administration of a hydroxamic acid into a joint cavity may first be proposed as an attempt to solve the above-described problems. However, frequent administration is required in order to maintain the local concentration of the hydroxamic acid; for the patients of OA and RA who unavoidably receive administration of the hydroxamic acid over a long time period, such frequent administration is very disadvantageous. The use of a so-called drug delivery system which restrictively localizes the hydroxamic acid at the target site may be proposed as alternative method. However, no methods for restrictively localizing or retaining the administered hydroxamic acid within the morbid joint have been established in the prior arts.

As mentioned above, although hydroxamic acids have excellent pharmacological properties, there still remain problems to be solved before they can be clinically applied as a therapeutic agent for chronic diseases such as OA and RA.

[0008]

Meanwhile the intraarticular injection of hyaluronic acid (hereinafter also referred to as "HA") and crosslinked product thereof (hereinafter also referred to as "HA formulation" as the general term for hyaluronic acid and its crosslinked product) currently finds extensive clinical application to joint diseases, especially OA and scapulohumeral periarthritis.

[0009]

Hyaluronic acid (HA) is an endogenous polysaccharide constituted by repeating units of N-acetylglucosamine and glucronic acid and, as the major component constituting the synovial fluid, it plays an important role in retaining the visco-elasticity of the synovial fluid, the load absorption function and the lubrication function. Furthermore, in the cartilage matrix, HA binds to cartilage proteoglycan to form a polymer called aglycan and plays a central role in maintaining the water retaining ability and viscoelasticity of the cartilage matrix.

[0010]

It is said that as a lubricant and also by enhancing the HA production in joints and the like, HA formulations generally have an effect to ease the disorder of joint

functions, although they do not inhibit MMPs. HA has a strong affinity to the extracellular matrix, since HA is inherently a constituent of the extracellular matrix, and in addition, HA has high visco-elasticity in itself; accordingly, HA is characteristically localized within the joint cavity for a long time period after it is injected into the joint cavity. In fact, in an experiment using ^{14}C labeled HA, it has been reported that the ^{14}C labeled HA as administered into a rabbit knee joint cavity is distributed to synovial fluid, synovial membrane tissue, the surface layer of articular cartilage and the like and it takes at least three days before the HA disappears from those tissues. Furthermore, it is said that HA does not undergo degradation in the synovial fluid and is partially degraded in the synovial membrane tissue and the articular cartilage but most of the HA slowly transfers into blood through the synovial membrane and decomposes into lower molecular substances in the liver.

[0011]

In addition, if a drug is bound to HA formulation before it is administered to a living body, it is expected that the drug is retained together with the HA formulation at a specific site for a long period of time and the duration of the drug action at the specific site is remarkably prolonged as compared to the case of administrating the drug alone. Furthermore, it is expected that by such an effect the dosage of the drug and the frequency of drug administration can be remarkably reduced

as compared to the conventional administrating method, resulting in greatly relieved side effects.

[0012]

So far, as HA-drug conjugates there are known an interferon/hyaluronic acid conjugate as described in Japanese Patent Publication (Kokai) No. Hei 5-85942/1993, a hyaluronic acid/anticancer agent conjugate as described in WO92/06714 Publication, a hyaluronic acid/corticosteroid conjugate as described in Japanese Patent Publication (Kokai) No. Sho 62-64802/1987, and a hyaluronic acid/antibiotic conjugate as described in Japanese Patent No. 2701865 and the like.

[0013]

However, in most of those cases, the effect of the drug is exhibited only after the drug is liberated from HA by decomposition of HA into lower molecular substances or by hydrolysis of the bond between HA and the drug, and taken up by the target cells or tissues. Accordingly, for joint cavities where almost no HA undergoes decomposition it is important to develop conjugates where the drug can exhibit medical effect even in the form of the conjugate with HA.

[0014]

[Problems to be Solved by the Invention]

One object of the present invention is to provide a covalent conjugate of an MMP inhibitor, particularly a matrix metalloprotease inhibitor capable of retaining a hydroxamic acid in a joint cavity, and hyaluronic acid, a

derivative thereof or a salt thereof.

Another object of the present invention is to provide a method for preparing the above described covalent conjugate.

Still another object of the present invention is to provide a pharmaceutical composition containing the above described covalent conjugate.

[0015]

[Means for Solving the Problems]

The present inventors have noted that there is a case in which a hydroxamic acid having MMP inhibiting activity has been proved to maintain the bindability to MMPs even when it is coupled to agarose which is one of artificial polysaccharides, and that all MMPs that have ever been discovered are exhibiting their enzymatic functions extracellularly or on the surface layer of cells. As the result of strenuous investigations made to solve the above described problems, the present inventors have found that a covalent conjugate, for example, a covalent conjugate of hydroxamic acid and HA formulation, prepared by allowing an MMP inhibitor to chemically bind to HA, an HA derivative or an salt thereof has possibility to exhibit MMP inhibition even in the conjugate form. The present invention has been achieved on the basis of this finding.

[0016]

In addition, the present inventors have found that similar to HA formulations, the covalent conjugate of an MMP inhibitor and HA, a derivative or a salt thereof which

was administered into joint cavity remained in the joint cavity for a long period of time, thereby reducing systemic side effects accompanying the MMP inhibitor and maintaining the medical effect of HA as the therapeutic agent for joint diseases; in other words, the present inventors have found that since the synergistic medicinal efficacy can be expected to manifest in the local site, the conjugate can be a pharmaceutical composition having improved biological utility. The present invention has been achieved on the basis of this finding.

[0017]

Thus, according to a first aspect of the present invention, there is provided a covalent conjugate of (1) at least one matrix metalloprotease inhibitor and (2) hyaluronic acid, a hyaluronic acid derivative or a salt thereof.

[0018]

In another mode of the covalent conjugate of the present invention, the matrix metalloprotease inhibitor binds to hyaluronic acid, the hyaluronic acid derivative or the salt thereof via a spacer.

In the covalent conjugate of the present invention, the weight ratio of the matrix metalloprotease inhibitor to the entire covalent conjugate is not particularly limited but is preferably 0.01 to 50%, particularly preferably 0.1 to 10%.

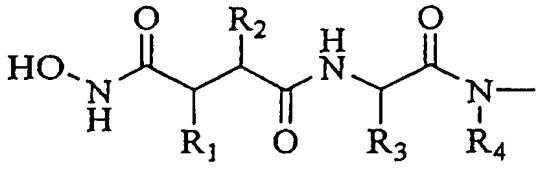
[0019]

In the covalent conjugate of the present invention,

the matrix metalloprotease inhibitor is preferably a hydroxamic acid residue.

The matrix metalloprotease inhibitor is particularly preferably a hydroxamic acid residue represented by the general formula (1),

[Formula 3]



(1)

wherein

R_1 is a hydrogen atom, a hydroxyl group or a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_2 is a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_3 is a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms which may be substituted with a cycloalkyl group, an aryl group or a heterocyclic group; and

R_4 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[0020]

In the covalent conjugate of the present invention, if there exists a spacer between the matrix metalloprotease inhibitor and the hyaluronic acid component, the spacer is particularly preferably represented by the general formula

(2),

$$-R_5-R_6-R_7-R_8- \qquad (2)$$

wherein

R_5 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms;

R_6 is a methylene group or an imino group, both of which may be substituted with a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms, or an oxygen atom;

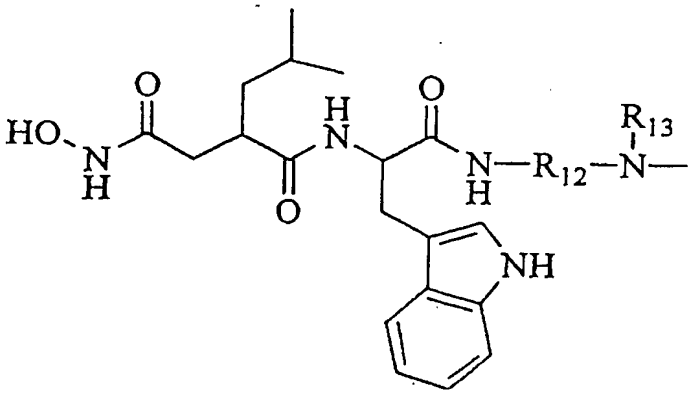
R_7 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms; and

R_8 is an oxygen atom, a sulfur atom or NR_9 wherein R_9 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[0021]

In the covalent conjugate of the present invention, particularly preferred examples of the conjugate of a matrix metalloprotease inhibitor and a spacer are represented by the general formula (3),

[Formula 4]



(3)

wherein

R_{12} is a straight-chain or branched-chain alkylene group having 3 to 21 carbon atoms or -(alkylene group having 1 to 8 carbon atoms)-O-(alkylene group having 1 to 8 carbon atoms)-; and

R_{13} is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[0022]

Furthermore, when the covalent conjugate of the present invention is administered to a living body, the matrix metalloprotease inhibitor, which is in the form of the conjugate with hyaluronic acid, a hyaluronic acid derivative or a salt thereof, can inhibit matrix metalloproteases.

[0023]

According to a second aspect of the present invention, there is provided a method for preparing the covalent conjugate of the present invention which comprises binding a site of a matrix metalloprotease inhibitor that does not affect the activity of the inhibitor to a carboxylic group, a hydroxyl group or a functional group at the reducing end of hyaluronic acid, a hyaluronic acid derivative or a salt thereof by direct chemical reaction or via a spacer. Thus, in the above described preparation method, a carboxylic group, a hydroxyl group or a functional group at the reducing end of hyaluronic acid, a hyaluronic acid derivative or a salt thereof is bound to a

site of a matrix metalloprotease inhibitor that does not affect the activity of the inhibitor either by direct chemical chemical reaction or via a spacer; binding via a spacer is performed in expectation of the possibility that at the time of binding reaction, the spacer is allowed to react with HA, a HA derivative or a salt thereof without being sterically affected by the MMP inhibitor by virtue of the space to be created between the MMP inhibitor and the reaction point at the distal end of the spacer and/or that in a conjugate, by virtue of the space to be created between the MMP inhibitor and HA, a HA derivative or a salt thereof, MMP will come sufficiently close to the MMP inhibitor without being sterically effected by the HA, HA derivative or salts thereof, thus the MMP inhibiting activity of the MMP inhibitor is maintained even in the conjugate form.

[0024]

According to a third aspect of the present invention, there is provided a pharmaceutical composition comprising the covalent conjugate of the present invention.

The pharmaceutical composition of the present invention is particularly a therapeutic agent for joint diseases, more specifically, a therapeutic agent for osteoarthritis, rheumatoid arthritis or scapulo-humeral periarthritis.

[0025]

[Mode for Carrying out the Invention]

In the present invention, a matrix metalloprotease

(MMP) inhibitor means all substances that can inhibit the activity of any matrix metalloprotease derived from any living body (preferably mammals, particularly preferably humans) by, for example, binding thereto.

[0026]

More specifically, MMP inhibitors mean: compounds or proteins (including polypeptides) which exhibit inhibition of the enzymatic activity of MMPs by binding to zinc, which is the active center of the MMPs, via a functional group such as a carboxylic acid, a phosphoric acid, a thiol and a hydroxamic acid; and those which inhibit expression of the enzymatic activity of MMPs or proteolytic enzymes having both disintegrin and MMP-like domains in their molecules [for example, TNFα converting enzyme or a group of proteases belonging to a disintegrin/metalloprotease family (ADAM)]. These MMP inhibitors are characterized in that they exhibit, as the inhibiting activity, 50% or more suppression at any concentration of 10mg/ml or less in a method by S. C. Cruwys et al.(cited in Br. J. Pharmacol, 100, 631-635(1990)) in which collagen degradation is caused by cartilage cells or synovial cells, or a method by M. DiMartino et al.(cited in Inflamm. Res., 46, 211-215(1997)) in which TNFα is liberated from human peripheral leukocytes. MMPs inhibitors also include those inhibitors whose structural formulae are chemically modified, provided that such inhibitors exhibit any one of inhibiting activities in the above method of at least 45% of suppression at any concentration of 10 mg/ml or less.

[0027]

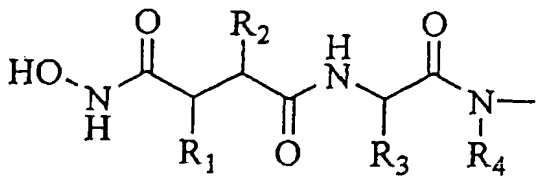
Non-limiting specific examples of MMP inhibitors include tetracycline compounds (such as tetracycline, doxycycline, minocycline and chemical modifications of tetracycline (for example, CMT 1 to 4, products of Collagenex)), TIMPs, and hydroxamic acids, and from the standpoints of the strength of MMP inhibiting activity and high specificity to MMPs, hydroxamic acids are preferred.

Examples of such MMP inhibitors are described in, for example, Japanese Patent Publication No. Hei 9-80825/1997, Japanese Patent No. 2736285 and Drug Discovery Today, 1, 16-26 (1996).

[0028]

A hydroxamic acid means a compound having an N-hydroxyamide group, and non-limiting specific examples of hydroxamic acid include AG-3340 (a product of Agouron), CDP-845 (a product of Zeneca), CGS-27023A (a product of Novartis), D5410 (a product of Chiro Science), L758354 (a product of Merck), CH-138 (a product of Chiro Science), Marimastat (registered trademark, a product of British Biotec), Galardin (registered trademark, a product of Glycomed), Ro31-9790 (a product of Roche), and Ro32-3555 (a product of Roche). Further, non-limiting specific examples of the hydroxamic acid residues in the covalent conjugates of the present invention include, for example, hydroxamic acid residues represented by the general formula (1),

[Formula 5]



(1)

wherein

R_1 is a hydrogen atom, a hydroxyl group or a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_2 is a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms;

R_3 is a straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms which may be substituted with a cycloalkyl group, an aryl group or a heterocyclic group; and

R_4 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[0029]

In the definition of the hydroxamic acid residues of the MMP inhibitors represented by the general formula (1), non-limiting specific examples of R_1 include a hydrogen atom, a hydroxyl group, a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, an isobutyl group, a tert-butyl group, an n-pentyl group, an n-hexyl group, an n-heptyl group, and an n-octyl group, and a hydrogen atom is preferred.

[0030]

Non-limiting specific examples of R_2 include a methyl

group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, an isobutyl group, a tert-butyl group, an n-pentyl group, an n-hexyl group, an n-heptyl group, and an n-octyl group, and an isobutyl group is preferred.

[0031]

Non-limiting specific examples of the alkyl group component of the straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms in the straight-chain or branched-chain alkyl group having 1 to 8 carbon atoms which may be substituted with a cycloalkyl group, an aryl group or a heterocyclic group in R_3 include a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, an isobutyl group, a tert-butyl group, an n-pentyl group, an n-hexyl group, an n-heptyl group, and an n-octyl group, and preferred are a methyl group, an isobutyl group and a tert-butyl group.

[0032]

Further, non-limiting specific examples of the cycloalkyl group, the aryl group or the heterocyclic group which may be present on the above described alkyl groups include cycloalkyl groups having 3 to 10 carbon atoms, preferably 5 to 7 carbon atoms (such as a cyclopentyl group, a cyclohexyl group, or a cycloheptyl group); aryl groups having 6 to 20 carbon atoms, preferably 6 to 14 carbon atoms (such as a phenyl group, a p-hydroxyphenyl group, or a naphthyl group) which may have a substituent such as a hydroxyl group and a methoxy group; and saturated or

unsaturated heterocyclic rings (such as a pyridyl group, a quinolyl group, or a 3-indolyl group, particularly preferably a 3-indolyl group) having 5 to 20 atoms, preferably 5 to 10 atoms, particularly preferably of 5, 6, 9 or 10 atoms and containing one or more hetero atoms which may be the same or different preferably 1 to 3 hetero atoms, particularly preferably one hetero atom, as selected from among a nitrogen atom, a sulfur atom and an oxygen atom.

[0033]

To give typical examples, R_3 is preferably a straight chain alkyl group having 1 to 5 carbon atoms which is substituted with an aryl group or a heterocyclic group and above all, particularly preferred are a benzyl group, a p-hydroxybenzyl group, and a 3-indolylmethyl group, and a 3-indolylmethyl group is the most preferred.

[0034]

Non-limiting specific examples of R_4 include a hydrogen atom, a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, an isobutyl group, and a tert-butyl group, and preferred is a hydrogen atom.

[0035]

The hydroxamic acid residues represented by the general formula (1) contain at least one asymmetric center carbon and as to each asymmetric center carbon, its absolute configuration may be the R-configuration or the S-configuration in the present invention.

[0036]

The weight ratio of the matrix metalloprotease inhibitor is preferably 0.01 to 50%, particularly preferably 0.1 to 10% based on the whole conjugate.

[0037]

In the present invention, "hyaluronic acid (HA)" means disaccharide polymers which have a weight average molecular weight of 100,000 to 10,000,000 and which are composed of glucuronic acid and N-acetylglucosamine, and a mixture of those polymers. From the standpoint of the strength in viscoelasticity, hyaluronic acid having a weight average molecular weight of 700,000 to 10,000,000 is preferred and hyaluronic acid having a weight average molecular weight of 1,000,000 to 10,000,000 is particularly preferred.

[0038]

In the present invention, "a hyaluronic acid derivative " means all substances that are derived from hyaluronic acid and which have a hyaluronic acid skeleton. Non-limiting specific examples of the hyaluronic acid derivative include:

(1) hyaluronic acid derivatives in which glucuronic acid and/or N-acetylglucosamine which are the sugar component has a reducing end;

(2) acetylated hyaluronic acid in which at least one hydroxyl group in hyaluronic acid is acetylated;

(3) derivatives of disaccharide polymers which have a weight average molecular weight of 100,000 to 10,000,000, which are composed of glucuronic acid and N-

acetylglucosamine and whole molecular weight is further increased by croslinking with formaldehyde (for example, Synvisc (registered trademark, a product of Biomatrix)); and

(4) derivatives obtained by allowing hyaluronic acid or the hyaluronic acid derivatives as described above in the present specification to bind, via a spacer or without a spacer, to at least one pharmaceutically effective component such as an anticancer agent (for example, an alkylating agent, a metabolic antagonist, and an alkaloid), an immunosuppressive agent, an anti-inflammatory agent(such as a steroid, a non-steroidal anti-inflammatory agent), an antirheumatic agent or an antibacterial agent (such as a β-lactam antibiotic, an aminoglycoside antibiotic, a macrolide antibiotic, a tetracycline antibiotic, a new quinolone antibiotic, a polypeptide antibiotic, and a sulfa agent).

[0039]

Non-limiting specific examples of salts of hyaluronic acid or the hyaluronic acid derivatives include a sodium salt, a potassium salt, a magnesium salt, a calcium salt and an aluminum salt.

[0040]

Although there is no limitation in the origin of HA, HA originated from bacteria such as *Actinomyces*, humans, pigs, and chicks can be used.

[0041]

Non-limiting specific examples of HA and salts

thereof include, for example, Suvenyl (registered trademark, Japan Roussel), Artz (registered trademark, Kaken Pharmaceutical Co., Ltd.), Opegan (registered trademark, Santen Pharmaceutical Co., Ltd.), Hyalgan (registered trademark, Fidia), Orthobisk (registered trademark, Anika Therapeutics), and Healon (registered trademark, Pharmacia & Upjohn). Further, HA and the salts thereof as described in the catalogs of various reagent makers such as Wako Pure Chemical Industries, Ltd. can also be included.

[0042]

In the conjugate of the present invention, a matrix metalloprotease inhibitor and hyaluronic acid, a hyaluronic acid derivative or a salt thereof are bound to each other via a spacer(s) or without any spacer. As the mode of binding between the matrix metalloprotease inhibitor and hyaluronic acid, a hyaluronic acid derivative or a salt thereof, covalent bonds such as an amide bond and an ether bond can be used in the absence of a spacer; or they are allowed to bind via a spacer(s). Preferably, the matrix metalloprotease inhibitor and hyaluronic acid, the hyaluronic acid derivative or the salt thereof are bound to each other via a spacer(s).

[0043]

When the matrix metalloprotease inhibitor and hyaluronic acid, the hyaluronic acid derivative or the salt thereof are bound to each other without a spacer, they bind covalently to each other at sites that do not adversely affect their activities. In addition, in the preferred

mode of the present invention in which the matrix metalloprotease inhibitor and hyaluronic acid, the hyaluronic acid derivative or the salt thereof are bound to each other via a spacer(s), the spacer(s) and MMP inhibitor or the spacer(s) and HA, the HA derivative or the salt thereof bind covalently to each other at sites that do not adversely affect the activities of the MMP inhibitor or HA, the HA derivative or the salt thereof.

[0044]

As to the MMP inhibitor, such sites that do not adversely affect their activities include, for example, an amino group, a carboxyl group, a hydroxyl group, and a thiol group. In a preferred mode of the present invention in which an MMP inhibitor is the hydroxamic acid residue represented by the general formula (1), such sites include a primary or secondary amino group positioned at the terminal end of the residue. As to HA, the HA derivative or the salt thereof, such sites include, for example, a hydroxyl group and a carboxyl group, preferably a carboxyl group.

[0045]

The type of the covalent bond between the MMP inhibitor and the HA, the HA derivative or the salt thereof, the type of the covalent bond between the spacer and the MMP inhibitor, and the type of the covalent bond between the spacer and the HA, HA derivative or salt thereof are not particularly limited; for example, an amide bond, an ether bond, an ester bond, and a sulfide bond can be

included.

The MMP inhibitor binding to HA, an HA derivative or a salt thereof is not necessarily limited to one type, and two or more different types of MMP inhibitors may be used. Further, one conjugate may have both a binding site interrupted by a spacer(s) and a binding site not interrupted by a spacer(s). Furthermore, spacers present in one conjugate are not necessarily the same.

[0046]

The type of the spacers is not particularly limited unless the activities of the MMP inhibitor and the HA, the HA derivative or the salt thereof are materially affected; non-limiting specific examples of the spacers include a spacer represented by the general formula (2),

$$-R_5-R_6-R_7-R_8- \qquad (2)$$

wherein

R_5 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms;

R_6 is a methylene group or an imino group which may be substituted with a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms or an oxygen atom;

R_7 is a straight-chain or branched-chain alkylene group having 1 to 8 carbon atoms; and

R_8 is an oxygen atom, a sulfur atom or NR_9 (wherein R_9 is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

The spacer represented by the above described general

formula (2) binds to an MMP inhibitor at the R_5-end thereof and binds to HA, an HA derivative or a salt thereof at the R_8-end thereof.

[0047]

In the definition of the spacer represented by the general formula (2), non-limiting specific examples of R_5 include a methylene group, an ethane-1,2-diyl group, a propane-1,3-diyl group, a butane-1,4-diyl group, a pentane-1,5-diyl group, a hexane-1,6-diyl group, a heptane-1,7-diyl group, an octane-1,8-diyl group, a 2-methylpentane-1,3-diyl group, 2-methylbutane-1,4-diyl group, a 3-methylbutane-1,4-diyl group, a 3-methylpentane-1,5-diyl group, a 3-ethylpentane-1,5-diyl group, a 3-methylhexane-1,6-diyl group, a 4-methylhexane-1,6-diyl group, and a 4-methylheptane-1,7-diyl group, and preferred are an ethane-1,2-diyl group, a propan-1,3-diyl group, and a butane-1,4-diyl group.

[0048]

As to R_6, examples of the straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms in the methylene group or imino group which may be substituted with a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms include a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, and a tert-butyl group.

[0049]

To give typical examples, R_6 is preferably a methylene group which may be substituted with a straight-chain or

branched-chain alkyl group having 1 to 3 carbon atoms or an oxygen atom, and particularly preferred is a methylene group or an oxygen atom.

[0050]

Non-limiting specific examples of R_7 include a methylene group, an ethane-1,2-diyl group, a propane-1,3-diyl group, a butane-1,4-diyl group, a pentane-1,5-diyl group, a hexane-1,6-diyl group, a heptane-1,7-diyl group, an octane-1,8-diyl group, a 2-methylpentane-1,3-diyl group, a 2-methylbutane-1,4-diyl group, a 3-methylbutane-1,4-diyl group, a 3-methylpentane-1,5-diyl group, a 3-ethylpentane-1,5-diyl group, a 3-methylhexane-1,6-diyl group, a 4-methylhexane-1,6-diyl group, and a 4-methylheptane-1,7-diyl group, and preferred are an ethane-1,2-diyl group, a propane-1,3-diyl group, a butane-1,4-diyl group, etc.

[0051]

Non-limiting specific examples of R_8 include an oxygen atom, a sulfur atom, an imino group, a methylimino group, an ethylimino group, an n-propylimino group, an isopropylimino group, an n-butylimino group, a sec-butylimino group, an isobutylimino group, and a tert-butylimino group, and preferred is an imino group or a methylimino group, etc., and particularly preferred is an imino group.

[0052]

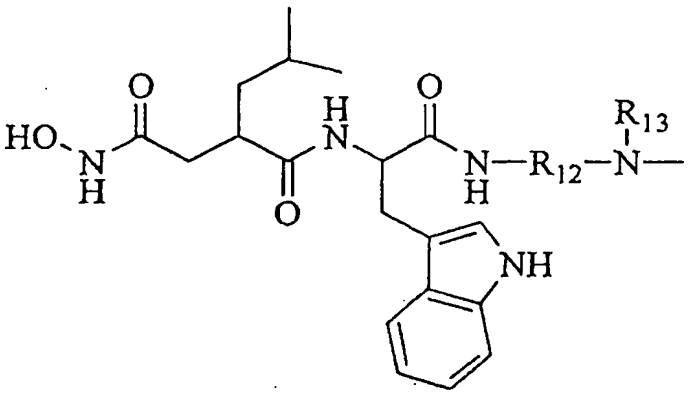
Preferred specific examples of the spacer represented by the general formula (2) include $-(CH_2)_4-NH-$, $-(CH_2)_5-NH-$, $-(CH_2)_6-NH-$, $-(CH_2)_7-NH-$, $-(CH_2)_8-NH-$, $-(CH_2)_9-NH-$, $-(CH_2)_{10}-NH-$,

$-(CH_2)_{11}-NH-$, $-(CH_2)_{12}-NH-$, $-(CH_2)_2-O-(CH_2)_2-NH-$, $-(CH_2)_3-O-(CH_2)_3-NH-$, and $-(CH_2)_4-O-(CH_2)_4-NH-$, etc.

[0053]

Furthermore, in the covalent conjugate in which a matrix metalloprotease inhibitor and hyaluronic acid, a hyaluronic acid derivative or a salt thereof are bound to each other via a spacer(s), preferred non-limiting specific examples of the conjugate of a matrix metalloprotease inhibitor and the spacer(s) include conjugates represented by the general formula (3),

[Formula 6]



(3)

wherein

R_{12} is a straight-chain or branched-chain alkylene group having 3 to 21 carbon atoms or -(alkylene group having 1 to 8 carbon atoms)-O-(alkylene group having 1 to 8 carbon atoms); and

R_{13} is a hydrogen atom or a straight-chain or branched-chain alkyl group having 1 to 4 carbon atoms.

[0054]

The hydroxamic acid residue moiety in the conjugates represented by the general formula (3) is the same as the preferred example of the MMP inhibitor represented by the general formula (1).

Further, non-limiting specific examples of R_{12} include an ethane-1,2-diyl group, a propane-1,3-diyl group, a butane-1,4-diyl group, a pentane-1,5-diyl group, a hexane-1,6-diyl group, a heptane-1,7-diyl group, an octane-1,8-diyl group, a nonane-1,9-diyl group, a decane-1,10-diyl group, an undecane-1,11-diyl group, a dodecane-1,12-diyl group, a 2-methylpentane-1,3-diyl group, a 2-methylbutan-1,4-diyl group, a 3-methyl-butane-1,4-diyl group, a 3-methylpentane-1,5-diyl group, a 3-ethylpentane-1,5-diyl group, a 3-methylhexane-1,6-diyl group, a 4-methylhexane-1,6-diyl group, a 4-methylheptane-1,7-diyl group, $-(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_3-O-(CH_2)_3-$, and $-(CH_2)_4-O-(CH_2)_4-$, etc., and preferred are a butane-1,4-diyl group, a pentane-1,5-diyl group, a hexane-1,6-diyl group, a heptane-1,7-diyl group, an octane-1,8-diyl group, a nonane-1,9-diyl group, a decane-1,10-diyl group, an undecane-1,11-diyl group, a dodecane-1,12-diyl group, $-(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_3-O-(CH_2)_3-$, and $-(CH_2)_4-O-(CH_2)_4-$, etc. Non-limiting specific examples of R_{13} include a hydrogen atom, a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-butyl group, a sec-butyl group, an isobutyl group, and a tert-butyl group, etc., and preferred are a hydrogen atom and a methyl group, etc., and particularly preferred is a hydrogen atom.

[0055]

Methods for preparing the covalent conjugate of the present invention include, for example, binding by chemical reaction a site (for example, an amino group, a carboxyl group, a hydroxyl group, a thiol group or the like) which does not affect the activity of an MMP inhibitor to a carboxyl group, a hydroxyl group or an aldehyde group originating from the reducing end of HA, an HA derivative or a salt thereof. This reaction can be carried out by known techniques (as described in "Shinseikagaku Jikken Koza (A New Course in Experimental Biochemistry), Vol.1, Proteins I" (Tokyo Kagakudojin), "Tanpakushitsu Koso no Kiso Jikken Hou (Basic Experimental Methods for Proteins and Enzymes)" (Nankodo) and the like).

Specific examples are as follows:

(1) a method for activating a carboxyl group in an MMP inhibitor or HA, an HA derivative or a salt thereof with the use of a dehydrative condensation agent to form an amide bond, an ester bond or a thioester bond;

(2) a method for activating a hydroxyl group in an MMP inhibitor with the use of cyanogen bromide and then binding the activated group to an amino group in HA, an HA derivative or a salt thereof, and a method for activating a hydroxyl group in HA, an HA derivative or a salt thereof with the use of cyanogen bromide and then binding the activated group to an amino group in an MMP inhibitor;

(3) a method for activating a hydroxyl group in an MMP inhibitor or HA, an HA derivative or a salt thereof with

the use of a epihalohydrin such as epichlorohydrin or a diepoxide such as 1,4-butanediol diglycidyl ether or a sulfonyl chloride such as tosyl chloride and tresyl chloride to form an ether bond, an imino bond or a sulfide bond; and

(4) a method for oxidizing a primary hydroxyl group formed by reducing the reducing end in HA, an HA derivative or a salt thereof to form an aldehyde group, and subjecting the resulting aldehyde to reductive alkylation with an amine in an MMP inhibitor.

Further, two or more of the above described methods (1) to (4) may be combined.

[0056]

In the method for activating a carboxyl group in an MMP inhibitor or HA, an HA derivative or a salt thereof with the use of a dehydrative condensation agent to form an amide bond, an ester bond or a thioester bond, condensation agents which are used in the general organic synthesis can be employed, and preferably carbodiimides, phosphoniums, uroniums and the like are used. Carbodiimides include, for example, non-water soluble carbodiimides such as diisopropyl carbodiimide and dicyclohexyl carbodiimde, and water soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; phosphoniums include, for example, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate and 7-azabenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; and uroniums include, for example,

O-benzotriazol-1-yl-N,N,N,N-tetramethyluronium hexafluorophosphate and O-7-azabenzotriazol-1-yl-N,N,N,N-tetramethyluronium hexafluorophosphate.

[0057]

Further, a reaction accelerating additive may be added to those condensation agents. Examples of such an additive include N-hydroxysuccinimide, p-nitrophenol, pentafluorophenol, 1-hydroxybenzotriazole, and 1-hydroxy-7-azabenzotriazole.

[0058]

Condensation by a water soluble carbodiimide is a non-limiting specific example of the method for activating carboxyl group in an MMP inhibitor or HA, an HA derivative or a salt thereof with the use of a dehydrative condensation agent to form an amide bond, an ester bond or a thioester bond. In this method, a carbodiimide is added to a 0.1 to 1% (weight/volume) HA aqueous solution, and subsequently an MMP inhibitor containing an amino group is added to the resulting solution and reaction is performed at 0°C to 35°C for 1 to 24 hours. During this reaction, an acid such as hydrochloric acid or phosphoric acid can be added to maintain the pH of the reaction solution at 4 to 6.

If an MMP inhibitor to be used has low water solubility, an aqueous solution containing 1 to 50% of an organic solvent (for example, N,N-dimethylformamide, N-methylpyrrolidone, dioxane, ethanol, pyridine or the like) can be used as the reaction solvent. In this case, an MMP inhibitor may first be added to the reaction system and the

carbodiimide may be added after confirming that the MMP inhibitor has dissolved.

[0059]

The following are non-limiting specific examples of the method for activating a hydroxyl group in an MMP inhibitor with the use of cyanogen bromide and then binding it to an amino group in HA, an HA derivative or a salt thereof and the method for activating a hydroxyl group in HA, an HA derivative or a salt thereof with the use of cyanogen bromide and then binding it to an amino group in the MMP inhibitor:

To an aqueous solution of HA, an HA derivative or a salt thereof, cyanogen bromide is added and reaction is performed at 0°C to 10°C for 5 to 30 minutes. During the reaction, the pH can be maintained at 10 to 12 with sodium hydroxide or a phosphate buffer solution or the like. Acetonitrile is then added to the reaction mixture to form a precipitate and excess cyanogen bromide is removed; the precipitate is reconstituted into an aqueous solution and an MMP inhibitor having an amino group is added to the solution and subjected to reaction at 4°C to 25°C for 1 to 24 hours. During the reaction, the pH of the reaction solution can be maintained at 8 to 10 with sodium bicarbonate, sodium hydroxide or the like.

[0060]

The following are non-limiting specific examples of the method for reducing the reducing end of HA, an HA derivative or a salt thereof to form a primary hydroxyl

group, oxidizing it to form an aldehyde group and subjecting the resulting aldehyde group to reductive alkylation with an amine in an MMP inhibitor:

Treatment with a reducing agent such as sodium borohydride and subsequent treatment with an oxidizing agent such as sodium periodate produces HA, an HA derivative or a salt thereof having an aldehyde group at the reducing end; to the obtained solution, an MMP inhibitor having an amino group is added; to the resulting mixture, sodium cyanoborohydride is added and reaction is performed at room temperature for 1 to 24 hours. During the reaction, the pH may be maintained at 4 to 6 by adding an acid such as acetic acid, hydrochloric acid, phosphoric acid or the like.

[0061]

In any of these condensation methods, the desired covalent conjugate can be obtained by adding an organic solvent such as ethanol and acetone to the reaction solution after the reaction to form a precipitate, which is then purified by a means such as alcohol precipitation, gel filtration, dialysis, or ion-exchange chromatography.

[0062]

If the conjugate of the present invention which comprises a matrix metalloprotease inhibitor bound to HA is to be applied as a drug, it is preferably used after being formulated into a pharmaceutical preparation together with a pharmaceutically acceptable excipient, stabilizer and the like.

The mode of administration of the drug or pharmaceutical composition is not particularly limited and may be oral or parenteral and may be systemic or local. In general, the pharmaceutical composition of the present invention is preferably administrated parenterally and locally, for example, intraarticularly, intraveneously, intramuscularly or subcutaneously as injenction, or percutaneously as a spraying agent, a topical cream or an ointment.

[0063]

The dosage of the pharmaceutical composition of the present invention can suitably be selected depending on the condition of the disease, age, and sex of the patient and the like; in the case of using it as injection, the amount of the conjugate as the effective ingredient generally ranges from 0.01 mg/body weight in kg/day to 100 mg/body weight in kg/day, preferably from 0.1 mg/body weight in kg/day to 10 mg/body weight in kg/day. The above described daily dosage per day may be administered in several divided portions a day or administered once a day or once in 2 to 28 days.

[0064]

[Example]

Example 1: Synthesis of MMP Inhibitor

(a) N-Benzyloxycarbonyl-1,4-diaminobutane

1,4-Diaminobutane (10g, 113 mmol) was dissolved in water/ ethanol(100 ml : 300 ml), and with stirring under cooling with ice a solution of benzyloxycarbonyl chloride

(19.35g, 113 mmol) in 1,2-dimethoxyethane (50 ml) was added dropwise over about 30 minutes. After a 2N sodium hydroxide aqueous solution (2 ml) was added, the resulting solution as such was stirred under cooling with ice for three hours and then stirred overnight at 4°C. After most of the solvent was distilled off under reduced pressure, the residue was dissolved in water and acidified with concentrated hydrochloric acid. The resulting solution was washed with chloroform (100 ml x 2) and then the aqueous layer was alkalized with a 2N sodium hydroxide aqueous solution, followed by extraction with chloroform. The resulting organic layer was washed with a saturated sodium chloride aqueous solution, dried over sodium sulfate and then the solvent was distilled off under reduced pressure to give 11.0 g of an oil. (Yield 44%)

^{1}H-NMR(270 MHz, $CDCl_3$): δ 1.4-1.5(4H, m), 2.7(2H, t), 3.2(2H, t), 5.1(2H, s), 7.3-7.4(5H, m)

MS: 222 (M^+)

[0065]

(b) N-9-Fluorenylmethyloxycarbonyl-L-tryptophan-N-(4-N-benzyloxycarbonylaminobutyl)amide

With stirring under cooling with ice, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC)(1.12 g, 5.85 mmol) was added to a solution (20 ml) of N-9-fluorenylmethyloxycabonyltryptophan (2.22 g, 4.5 mmol) and 1-hydroxybenzotriazole (0.90 g, 5.85 mmol) in N,N-dimethylformamide (DMF) and stirred for one hour. To the reaction solution, the N-benzyloxycarbonyl-1,4-

diaminobutane (1g, 4.5 mmol) as obtained in (a) above was added, the resulting mixture as such was stirred under cooling with ice and then stirring was continued overnight at room temperature. After most of the solvent had been distilled off under reduced pressure, the residue was dissolved in chloroform (100 ml) and washed with a 0.5N hydrochloric acid aqueous solution (40 ml x 2), a saturated sodium bicarbonate aqueous solution (50 ml) and a saturated sodium chloride aqueous solution (50 ml). The organic layer was dried over anhydrous sodium sulfate and then concentrated. The resulting residue was purified by silica gel column chromatography using chloroform/methanol as the eluting solution to obtain 2.1 g of colorless powder. (Yield 74%)

^{1}H-NMR(270 MHz, $CDCl_3$): δ 2.2-3.4(10H, m), 4.2(1H, t), 4.3-4.5(3H,m), 5.1(2H, s), 7.0-8.0(18H, m)

[0066]

(c) L-Tryptophan-N-(4-N-benzyloxycarbonylaminobutyl)amide

The condensation product (2.1 g) as obtained in (b) above was dissolved in DMF (50 ml), and piperidine (3 ml) was added to the solution; the resulting solution was stirred at room temperature for 30 minutes. After most of the solvent was distilled off under reduced pressure, the residue was purified by silica gel column chromatography using chloroform/methanol as the eluting solution to obtain 1.0 g of a transparent oil. (Yield 74%)

^{1}H-NMR(270 MHz, $CDCl_3$): δ 1.4(4H,m), 3.0-3.4(6H,m), 3.7(1H,m), 5.1(2H,s), 7.0-7.7(9H,m)

MS:408(M^+)

[0067]

(d) [4-(N-Benzyloxyamino)-2-isobutylsuccinyl]-L-tryptophan-N-(4-N-benzyloxycarbonylaminobutyl)amide:(1)

L-Tryptophan-N-(4-N-benzyloxycarbonylaminobutyl)amide (1.18 g, 2.9 mmol) was dissolved in DMF (30 ml), and with stirring under cooling with ice, 4-(N-benzyloxyamino)-2-isobutylsuccinic acid (732 mg, 2.6 mmol) as synthesized according to a known method [for example, Japanese Patent Publication (Kokai) No. Hei 6-145148/1994, etc.] and EDC (552 mg, 2.9 mmol) were successively added; the reaction temperature was set between the temperature of cooling with ice and that of cooling with water, and stirring was continued for three days. The reaction solution was concentrated under reduced pressure and diluted with chloroform; the chloroform layer was successively washed with 0.1N hydrochloric acid, water, a saturated sodium bicarbonate aqueous solution and a saturated sodium chloride aqueous solution, and dried over sodium sulfate. After filtration, the filtration residue and the aqueous layer were re-extracted with ethyl acetate; the ethyl acetate layer and the chloroform layer were combined and concentrated under reduced pressure. The obtained crude product was subjected to silica gel chromatography purification (WAKO, C-200, eluting solvents: chloroform and a 1 : 1 mixture of chloroform and acetone); the resulting fractions were collected and concentrated under reduced pressure and dried to obtain 1.20 g (68%) of the title

compound (1).

MS: 670 ($M+H^+$)

[0068]

(e) 4-(N-hydroxyamino)-2(R)-isobutylsuccinyl]-L-tryptophan-N-(4-N-aminobutyl)amide:(2)

[4-(N-hydroxyamino)-2(S)-isobutylsuccinyl]-L-tryptophan-N-(4-N-aminobutyl)amide:(3)

[4-(N-Benzyloxyamino)-2-isobutylsuccinyl]-L-tryptophan-N-(4-N-benzyloxycarbonylaminobutyl)amide (1) (1.20 g, 1.8 mmol) was dissolved in 50 ml of methanol and catalytically reduced with 140 mg of 10% Pd/C under an atmospheric pressure of hydrogen for 16 hours. The reaction solution was filtered with celite and then concetrated under reduced pressure. The obtained crude product was subjected to reverse phase HPLC (column: YMC-Pack, ODS, 250 mm x 20 mm I.D., eluting solvent: a 0.1% trifluoroactic acid (TFA)-containing water/acetonitrile system, flow rate: 10 ml/min) and respective diastereomers were recovered and purified and freeze-dried to obtain 283 mg of a TFA salt of the title compound (2) (peak at the hydrophilic side) and 493 mg of a TFA salt of the title compound (3) (peak at the hydrophobic side), respectively.

[0069]

(2):

^{1}H-NMR(270 MHz, CD_3OD):0.70(3H,d,J=6Hz), 0.77(3H,d,J=6Hz), 1.02-1.53(7H,m), 2.12(1H,dd,J=14,5Hz), 2.29(1H,dd,J=14,9Hz), 2.59-2.68(1H,m), 2.80-2.85(2H,m), 3.10-3.36(4H,m), 4.49-4.58(1H,m), 6.96-7.09(3H,m), 7.30(1H,d,J=8Hz),

7.57(1H,d,J=8Hz), 7.95-8.04(2H,m)

MS:446($M+H^+$)

[0070]

(3):

^{1}H-NMR(270 MHz,CD_3OD):0.51(3H,d,J=6Hz), 0.56(3H,d,J=6Hz), 0.63-0.92(2H,m),1.11-1.21(1H,m), 1.56-1.58(4H,m), 2.02(1H,dd,J=15,2Hz),2.31(1H,dd,J=15,11Hz),2.48-2.60(1H,m), 2.86-3.45(6H,m), 4.64-4.72(1H,m), 6.91-7.04(3H,m), 7.27(1H,d,J=8Hz), 7.54(1H,d,J=8Hz), 7.97-8.08(2H,m)

MS:446($M+H^+$)

[0071]

Example 2: Conjugate Synthesis Example 1

To 70 mg of an MMP inhibitor as obtained in Example 1(2), 0.49 ml of N-methylpyrrolidone and 0.01 ml of pyridine were added to dissolve the inhibitor; the pH of the solution was adjusted to 4.7 with 0.045 ml of 1M hydrochloric acid and water and its whole volume was adjusted to 1 ml. The resulting solution was added to 5 mg of sodium hyaluronate (derived from human umbilical cord, molecular weight 800,000 to 1,200,000, Seikagaku Kogyo Co.,Ltd.) to form a uniform mixture. After reconfirming that the pH was 4.7, the reaction solution was added with 10 mg of EDC under cooling with ice and stirred for 30 minutes, and further stirred at room temperature for 15 hours.

To the reaction solution, 1 ml of 0.1M sodium bicarbonate and 6 ml of ethanol were added to form a precipitate which was then purified by repeating the

alcohol precipitation method three times (the method comprising the steps of dissolving the precipitate in 1 ml of a 0.2M sodium chloride aqueous solution, effecting precipitation with 3 ml of ethanol and centrifuging the precipitate), thus producing 4.3 mg of a conjugate.

The bonding amount calculated from the UV absorption at 279 nm derived from an indole ring was 0.84% by weight. This corresponds to that 0.76% of the carboxyl group reacted.

[0072]

Example 3: Conjugate Synthesis Example 2

To 70 mg of an MMP inhibitor obtained in Example 1 (3), 0.49 ml of N-methylpyrrolidone and 0.01 ml of pyridine were added to dissolve the inhibitor; the pH of the solution was adjusted to 4.7 with 0.045 ml of 1M hydrochloric acid and water and its whole volume was adjusted to 1 ml. The resulting solution was added to 5 mg of sodium hyaluronate (derived from human umbilical cord, molecular weight 800,000 to 1,200,000, Seikagaku Kogyo Co.,Ltd.) to form a uniform solution. After reconfirming that the pH was 4.7, the reaction solution was added with 10 mg of EDC under cooling with ice and stirred for 30 minutes, and further stirred at room temperature for 20 hours.

To the reaction solution, 1 ml of 0.1M sodium bicarbonate and 6 ml of ethanol were added to form a precipitate which was then purified by repeating the alcohol precipitation method three times (the method

comprising the steps of dissolving the precipitate in 1 ml of a 0.2M sodium chloride aqueous solution, effecting precipitation with 3 ml of ethanol and centrifuging the precipitate), thus producing 3.5 mg of a conjugate. The bonding amount calculated from the UV absorption at 279 nm derived from an indole ring was 1.1% by weight. This corresponds to that 1.0% of the carboxyl group reacted.

[0073]

[Advantageous Effects of the Invention]

The covalent conjugate of the present invention is retained, for example, in a joint cavity where it is administered for as long a period of time as conventional HA formulations, and hydroxamic acids therein which are bound to HA, a HA derivative or a salt thereof can inhibit local MMP. Therefore, localization and prolongation of the action of MMP inhibitor at the administration site (for example, joints such as knees and shoulders etc.) as well as reduction of the frequency of administration which could never be accomplished with the existing technology are possible, and it is expected to reduce the adverse side effects of MMP inhibitors considerably as compared to the conventional method of systemic administration.

[0074]

Additionally, since both the drug component of HA, a HA derivative or a salts thereof and a MMP inhibitor component can exhibit their respective pharmaceutical effects without being dissociated or decomposed, it is expected to obtain the synergistic pharmaceutical effects.

[0075]

For these reasons, the covalent conjugate of the invention features enhanced pharmaceutical utility both as a MMP inhibitor (such as hydroxamic acid) and as HA or an HA derivative or a salt thereof, for example, as a drug with enhanced ability to suppress joint destruction; the conjugate is therefore anticipated to be an effective drug for treating osteoarthritis, rheumatoid arthritis or scapulohumeral periarthritis.

[Name of Document] Abstract

[Abstract]

[Problems] To provide a covalent conjugate of a matrix metalloprotease inhibitor and hyaluronic acid, a hyaluronic acid derivative or a salt thereof which can retain the matrix metalloprotease inhibitor, particularly hydroxamic acid in joint cavities.

[Means for Solving] A covalent conjugate of at least one matrix metalloprotease inhibitor and hyaluronic acid, a hyaluronic acid derivative or a salt thereof; a method for preparing the above described covalent conjugate which comprises binding a site of the matrix protease inhibitor which does not affect the activity of a matrix metalloprotease inhibitor to a carboxyl group, a hydroxyl group or a functional group at the reducing end of hyaluronic acid, a hyaluronic acid derivative or a salt thereof by direct chemical reaction or via a spacer; and a pharmaceutical composition containing the above described covalent conjugate.

[Selected Drawing] None.